Egypt. J. Chem. **Vol. 65,** No. SI: 13B pp. 195-201 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

The Effectiveness of Begonia Multangula Blume Leaf Ethanol Extract as Polymicrobial Antibiofilm on Catheters

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Abstract

The growth of catheter biofilm is associated with urinary tract nosocomial infections and causes an increase in mortality every year. *Begonia Multangula* Blume leaves are known have antibacterial activity but their antibiofilm activity has not been reported. This study aimed to determine the effectiveness of *Begonia Multangula* Blume extract in inhibiting polymicrobials in catheters. The research was conducted using the *microtiter broth* method. Antibiofilm activity was determined as the *minimum biofilm inhibitory concentration* ($MBIC_{50}$), the *minimum biofilm eradication concentration* ($MBEC_{50}$). Antibiofilm mechanism elucidated using *scanning electron microscopy* (SEM). Statistical analyzes were performed using ANOVA (P <0.05). *Begonia Multangula* blume leaf extract 1% v/v gave inhibitory activity of polymicrobial biofilm formation in the middle phase catheter of 65.23 % ± 0.01 and maturation phase of 60.44 % ± 0.01 almost equivalent to the control drugs chloramphenicol and nystatin 1 % v/v. The results provide evidence that the leaves of *Begonia Multangula* blume can eradicate 60% polymicrobial biofilms 63.55 % ± 0.01. Observations using SEM showed disruption the *Extracellular Polymeric Substances* (EPS) layer due to the test compound. The conclusion of this study, that *Begonia Multangula* blume leaf ethanol extract has the potential to be developed as a candidate for new antibiofilm drugs against polymicrobial biofilms on catheters.

Keyword: Antibiofilm, *Begonia multangula* Blume , Catheter, Infection, Polymicrobial Biofilm

1. Introduction

Microbes' development of biofilm forms is currently recognized as the primary mediator of infection, and it is estimated that 80% of all infections are caused by the formation of microbial biofilms [1]. Urinary catheters are now the most commonly used medical device worldwide, with an estimated more than 100 million urethral catheters sold annually [2], and more than 30 million urinary catheters inserted annually in the US alone [3]. While this simple device can be of considerable benefit to many people, its use undermines the natural defenses of the urinary tract. Thus management of catheter patients is often complicated by infections where biofilm formation is a crucial feature. Bacterial biofilms are groups of bacteria that adhere to surfaces or each other and are incorporated into their matrix [4]. Given the advantageous use of urinary catheters in modern medicine, it is not surprising that catheter-associated urinary tract infections (CAUTI) are one of the most common nosocomial infections [5]. Patients with catheter-associated bloodstream infection (CRBSI) [6]. The longer the catheter, the more likely bacteriuria will occur. The biofilm that forms on indwelling urinary catheters and other

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Receive Date: 28 January 2022, Revise Date: 30 March 2022, Accept Date: 06 April 2022

DOI: 10.21608/EJCHEM.2022.118622.5341

urinary devices is different from the biofilm on non-urinary devices [7]. Their strong absorptive capacity allows biofilms to grow even in highly nutrient-deficient environments [8]. Bacterial pathogenicity is enhanced by biofilm formation (Muslim *et al.*, 2021). Biofilm formation can be stimulated by the presence of serum and saliva in the environment [9]. Many other species that infect the urinary tract and catheters form extensive biofilms on long-term catheters . Although not responsible for clinical emergencies, significant increases often occur in biofilm cells, causing bacteria to become resistant to antibacterial agents [10]

Currently, the use of traditional medicine in Indonesia has progressed quite rapidly. Traditional medicines are now starting to be used again by the community as an alternative treatment, although modern medicines or synthetic medicines are still circulating in the market [11]. Although traditional medicines derived from plants and natural ingredients also have side effects, the level of danger and risk of long-term use is much lower and has great economic value than chemical drugs drugs [12]. In general, *Begonia Multangula* blume leaves have glossy, dark green (sometimes almost black) pubescent leaves, rarely with silver or light green spots [13], with growing habitats karst limestone or sandstone forest grows on vertical limestone cliffs under the shade [14]. Phytochemical test results showed that *Begonia Multangula* blume contains phenolic compounds, flavonoids, steroids, terpenoids, and alkaloids. *Begonia Multangula* blume positive contains saponins, alkaloids, tannin compounds, phenolic compounds, ethyl palmitate, palmitic acid, ethyl linolenic acid, and acetol [15].

The studies outcomes by using [16] and [17] display that *Begonia multangula* Blume has antibacterial hobby towards *Staphylococcus aureus* and *Escherichia coli*. Extracts from the stalks and leaves of *Begonia multangula* Blume were also known to have antibacterial interest in opposition to *P. gingivalis*.

The type of activity formed was bactericidal activity, which indicated that the extract completely inhibited bacterial growth [18]. Biofilm growth on catheters is associated with urinary tract nosocomial infections and causes an increase in mortality every year. Infections with associated deaths occurring in both fungal and bacterial cases, the opportunity for appropriate antibiotic therapy is hampered by antibiotic resistance [19]. Begonia *Multangula* blume leaves have antibacterial activity, but their antibiofilm activity has never been reported. This study aims to determine the effectiveness of *Begonia Multangula* blume extract in inhibiting and eradicating polymicrobial in catheters

2. Experimental

a. Material

The material used is the ethanol extract of *Begonia multangula* Blume. Other ingredients included the subsequent: crystal violet (Merck, Germany), ethyl acetate (Merck, Germany), coronary heart-brain infusion (Oxoid) (Merck, Germany), RPMI 1640 (Sigma-Aldrich), catheter, ninety five% ethanol (Merck, Germany). Germany), nystatin, chloramphenicol (Sigma-Aldrich, Germany).

b. Equipment

Laminar Air glide, incubator (IF-2B) (Sakura, Japan), micropipette pipetman (Gilson, France), multichannel micropipette (Socorex, Switzerland), microplate flat-bottom polystyrene 24 well (Iwaki, Japan), microtiter plate reader (Optic Ivymen gadget 2100-C, Spain), spectrophotometry (Genesys 10 UV Scanning, 335903) (Thermo scientific Spectronic, united states of america), autoclave (Sakura, Japan), incubator with orbital shaker S1500 (Stuart, united kingdom), analytical balance (AB204 -5, Switzerland).

c. Preparation of fungi and bacteria for assay

Staphylococcus aureus (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) bacteria were all grown within 24 hours at 37ºC in BHI (Brain Heart Infusion) media. Meanwhile, *Candida albicans* (ATCC 10231) was grown for 72 hours at 37ºC in *Sabouraud Dextrose Broth* (SDB). The *optical density* 600 (OD_{600}) of the microbial culture was adjusted to 0.1 equivalent to the McFarland standard 0.5-1.5 x 108 CFU/mL), and then diluted in a new growth medium to OD_{600} 0.01 for bacteria and OD_{520} 0.38 for *C. albicans*.

d. Catheter Biofilm Test

In the biofilm inhibition test, the catheter was cut to a length of one centimeter then sterilized in 70% ethanol, allowed to dry and the catheter was inserted into the wells [20]. A total of 100 µL of media containing bacterial suspension, normal human urine, and test compounds were added to each wells microtiter plate that already contained a catheter, then incubated at a temperature of ± 37ºC for 24 hours for the middle phase and 48 hours for the mature phase. After the formation of the biofilm, the suspension in the microplate was discarded. Then the plate was washed with distilled water three times and dried at room temperature for 5 minutes to remove the remaining water. Meanwhile, in the biofilm eradication activity test, each wells that had been catheterized were put in media containing a suspension of bacteria and normal human urine for 48 hours at 37ºC. After the incubation period, the plates

were washed using 150 µL of sterile distilled water. A total of 100 µL of media containing Ethanol extracts of *Begonia multangula* Blume with concentration series (1%, 0.5%, 0.25%, 0.125% v/v) was added to each wells that had been washed and incubated for 48 hours at 37ºC.

The catheter was then scraped and transferred to a new plate and added 125 µL of 1% crystal violet solution into each well to color the biofilm that had formed, both dead cells and live cells which are also components of the biofilm [20]. Then incubated at room temperature for 15 minutes. After further incubation, the biofilm was washed with running water three times to remove the remaining crystal violet and 200 L of 96% ethanol was added to each well to dissolve the formed biofilm. As a positive control, the microbial suspension was used which was given chloramphenicol and nystatin with a concentration of 1% v/v. The test was carried out with three replications. The biofilm degradation results were read using a 595 nm *Optical Density* (OD) *microplate reader*. The OD value is then used to calculate the percent biofilm degradation in equation below:

$$= \frac{(OD_{\text{negative control mean}} - OD_{\text{test sample mean}})}{OD_{\text{negative control mean}}} \times 100\%$$

e. Documentation of biofilm structure using *Scanning Electron Microscopy* (SEM)

Biofilm observations using Scanning Electron Microscopy (SEM) were carried out at the UGM Integrated Research and Testing Laboratory (LPPT), which was modified from [20]. The cover slip was put into a 24 well round bottom polystyrene microtiter plate which contained the test suspension which had been given the same treatment as the biofilm formation inhibition test. After being incubated at 37°C for 24-48 hours, the cover slips were carefully washed three times with sterile distilled water, then fixed with 2.5% (v/v) glutaraldehyde in cacodylate buffer for ± 24 hours with the aim of killing. cells without changing the structure of the cells to be observed. Furthermore, dehydration using methanol was carried out for 30 minutes to reduce the water content so that it did not interfere with the observation process. The samples were then observed under *Scanning Electron Microscopy* (SEM) with a voltage of 10 kV.

f. Statistical analysis

Statistical analysis was performed using ANOVA and Normality test performed using the Shapiro – Wilk, with *P* values of 0.05 or less. The data were analyzed using the *Statistical Package for the Social Sciences* (SPSS).

3. Results And Discussion

a. Effect of *Begonia multangula* Blume ethanol extract on Polymicrobial Biofilms on catheters in the mid-phase (24 hours) and mature phase (48 hours)

Polymicrobial biofilm is a mixture of several bacterial species. In this study, we examined the potential of the antibiofilm of *Begonia multangula* Blume against biofilm inhibition of polymicrobial such as *S. aureus*, *P. aeruginosa*, *E. coli*, and *C. albicans*. Polymicrobial infections caused by a combination of microorganisms are responsible for significant mortality and morbidity [16]. The results also provide evidence of the activity of *Begonia multangula* Blume leaf extract in inhibiting the growth of polymicrobial biofilms on catheters in the mid and maturation phases.

In the middle phase, *Begonia multangula* Blume leaf extract 1% v/v gave an inhibitory activity of polymicrobial biofilm formation on the catheter of 65.23 % ± 0.01 ,$MBIC_{50}$ activity of Begonia (Multangula blume) leaf extract is around starting from0.25% v/v (**P*<0.05) and this was almost equivalent to the control drug chloramphenicol and nystatin, which was 63.55 % ± 0.01 (Figure 1). This is because these bacteria can easily form biofilms in just 24 hours. This inhibition can occur because the EPS matrix produced by polymicrobial biofilms in the intermediate phase is not yet complex and structured so that the ethanol extract of *Begonia multangula* Blume can inhibit the growth of EPS biofilms. This result by [15] statement which states that the active substance from the leaves of *Begonia multangula* Blume works as an antibacterial in which the surface tension of the bacterial cellular wall will be reduced and the permeability of the bacterial membrane can be broken.

In the maturation phase, there is a decrease in activity compared to the middle phase. This is because the biofilm growth time in this phase is longer and the biofilm defense system has formed a more dense and complex defense. Biofilm formation is generally considered to be a cyclic process consisting of phenotypically distinct stage. According to research by [22, 23] showed thickening of the biofilm can be caused by the number of bacterial components and increases the stability of bacterial species with one another. In this way, the extracellular matrix produced by one organism can contribute to collectively protecting other organisms in the biofilm [24].

The biofilm in the mature phase inhibition from Begonia extract gave mature activity of 60.44 ± 0.01and the effect is similar to the chloramphenicol and nystatin as the control groups. $MBIC_{50}$ activity of Begonia extract is around 0.05% v/v (**P*<0.05).In the

mature phase, the biofilm produces a thicker and more complex structure than the middle phase, it can be seen from the mucus layer produced in the mature phase which is denser and attached to the wells. Therefore, the biofilm in the maturation phase makes it difficult for antibiotics to penetrate the microbes wrapped in the biofilm due to the presence of an EPS matrix layer. Slow bacterial growth will increase EPS production because EPS is highly hydrated to prevent drying out in some natural biofilms. EPS may also contribute to the antimicrobial resistance properties of biofilms by inhibiting the bulk transport of antibiotics through the biofilm [25].

This result is by the statement of [26] where biofilms in the mature phase are more difficult to penetrate than biofilms in the middle phase. The above statement is also by the research of [27] which states that in the maturation phase, antimicrobial agents will have more difficulty penetrating biofilm defenses. This can be caused because the bacteria that grow in the biofilm are in a dormant phase so they can avoid environmental stress, one of which is due to the influence of antibiotics [28].

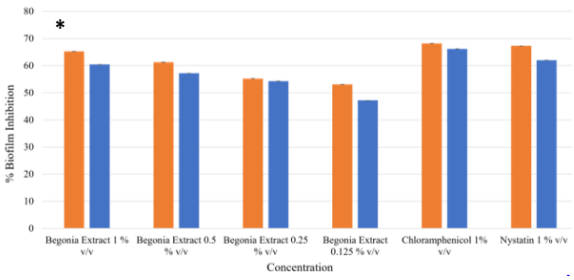


Figure 1. Inhibition percentage of extract *Begonia multangula* Blume (red bar: middle-phase, blue bar: maturation-phase (*P <0.05).

b. Eradication Activity of *Begonia multangula* Blume Ethanol Extract Against Polymicrobial Biofilm on catheters

Our results provide evidence that the ethanolic extract of *Begonia multangula* Blume is capable of eradicating polymicrobial biofilms composed of *S. aureus*, *P. aeruginosa*, *E.coli*, and *C. albicans*. is capable of eradicating polymicrobial biofilms composed of *S. aureus*, *P. aeruginosa*, *E. coli*, and *C. albicans*. These results indicate that the eradication activity decreased compared to the 24-hour phase biofilm inhibitory activity. This is because the EPS formed in this phase is very complex and structured and is categorized in the 4th stage of biofilm growth, where bacteria can communicate with one another and synergize in manipulating drug compounds that will penetrate the biofilm defenses formed by their communities. Wherefrom previous research also stated that biofilm is a complex three-dimensional structure consisting of living bacteria in an extracellular matrix or secreting polymeric substances containing polysaccharides, nucleic acids, and proteins. Infections caused by biofilms are difficult to eradicate because polymeric substances excreted in biofilms can increase the growth of resistant bacteria and prevent antibiotics from reaching these bacteria [29]. Biofilm dispersion is the final stage of biofilm development, where: actively escape from the biofilm in response to deteriorating conditions within the biofilm [30]. The activity of *Begonia multangula* Blume leaf extract was able to eradicate 60% of the polymicrobial biofilm on the catheter by 63.55 % ± 0.01, (Figure 2), with $MBEC_{50}$ activity of *Begonia multangula* Blume extract is around 1 % v/v (*P<0.05). These results provide very important information that the ethanolic extract of begonia can be used as one of the polymicrobial antibiofilm on catheters leading to their eradication.

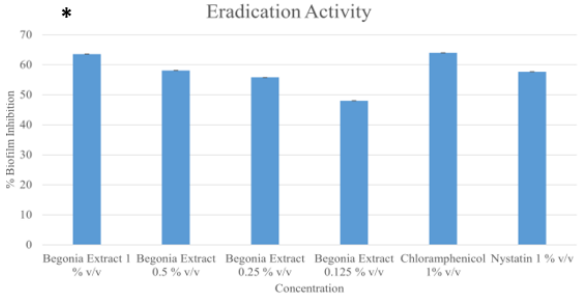
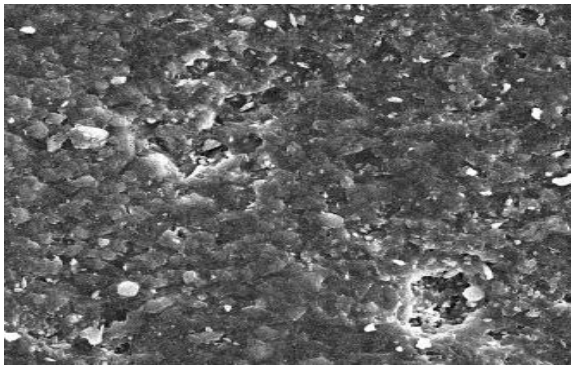


Figure 2. Inhibitory Effect of extract *Begonia multangula* Blume biofilm at eradication phase (*P <0.05).

c. *Scanning Electron Microscopy* (SEM) results on polymicrobial biofilms

The figure 3 shows a very complex cell density and a strong biofilm structure. In addition, the EPS matrix formed is very large. The SEM results showed that untreated polymicrobial biofilms showed dense, structured cell density and the formation of EPS produced by polymicrobial biofilms. In some literature, it is stated that bacteria synergistically form biofilms with other bacterial species, and physically and physiologically the biofilm structure is thick and strong [31]. Biofilm formation is one of the most effective forms of bacterial persistence on surfaces where nutrients are available or in living host tissues as humans or animals [32]. The biofilm formation process also involves several bacteria, including the QS system and the two-component regulatory system, both of which interact especially during the production of EPS [33]. Meanwhile, the SEM results of polymicrobial biofilms that had been given 0.5% v/v ethanol extract of *Begonia multangula* Blume showed shrinkage, damage to the EPS matrix, and cell lysis. This is due to an increase in cell membrane

permeability and degradation of mature biofilms by killing biofilms embedded in biofilms. cause cell damage.stated that the ethanol extract of Begonia was able to reduce the attachment of yeast cells. *Begonia multangula* Blume ethanol extract was able to damage the EPS matrix of the polymicrobial biofilm on the catheter and reduce the density of the polymicrobial biofilm cell [34].



(A)

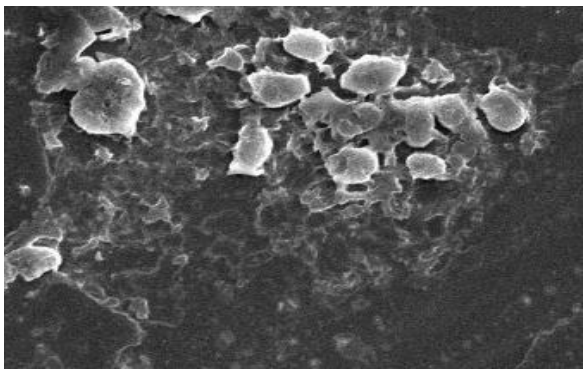


Figure 3. Polymicrobial biofilms of *S.aureus*, *P. aureginosa*, *E. coli*, and *C. albicans* on Catheters were taken using *Scanning Electron Microscopy* (EPS) with x1000 magnification. (A) before administration of *Begonia multangula* Blume ethanol extract, (B) after administration of *Begonia multangula* Blume ethanol extract

4. Conclusion

The Ethanol extract from the leaves of *Begonia multangula* Blume can inhibit the growth of polymicrobial biofilms and has eradication activity against polymicrobial biofilms on catheters. Therefore, the ethanol extract of *Begonia multangula* Blume has the potential to be developed as a candidate for new antibiofilm agents against polymicrobial biofilms on catheters.

5. Acknowledgment

Thank you to the Faculty of Pharmacy, Universitas Muhammadiyah Kalimantan Timur, for facilitating the completion of this research. Thanks also to Universitas Halu Oleo Kendari, and Universitas Mulawarman, Samarinda for their cooperation so that this research can run well.

6. Conflict Of Interest

The authors claim that they've no recognised competing economic hobbies or private relationships that could have appeared to persuade the paintings stated on this paper.

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